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AVIAN INFECTIOUS BRONCHITIS VIRUS (IBV): EFFECT OF VACCINE DOSES ON MUCOSAL IMMUNE RESPONSES AND PROTECTION AFTER CHALLENGE IN CHICKENS

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ABSTRACT

Scarce information is available on the effect of inappropriate dose of vaccines against IBV on the mucosal immune responses and how it may relate to incomplete protection. This study aims to investigate the effect of vaccine doses on the cell-mediated immune (CMI) and antibody responses of respiratory tract and their correlation to protection against IBV infection. Significantly reduced tracheal lesions and viral replication were found only for full-dose vaccinated group, at 5 dpi, while incomplete protection was seen for sub-dose vaccinated groups. Moreover, the full-dose vaccinated group showed higher and significant levels of lachrymal IgG and IgA anti-IBV antibodies, and early significant increase in the expression of CMI genes, specially CD8 and Granzyme A. Thus, administration of sub-doses of the IBV vaccine can reduce the CMI and antibody memory responses at the respiratory mucosal sites, leading to lower degrees of protection against IBV infection.

KEYWORDS: Infectious

INTRODUCTION

Avian infectious bronchitis virus (IB) is a worldwide infectious disease which causes significant economic losses for poultry industry. This disease could be efficiently prevented by attenuated vaccines when administered locally, but not by systemically inactivated vaccines, suggesting that respiratory mucosal immunity is essential for protection and its development may rely on direct interaction between mucosal surface and IBV (WANG *et al.*, 2006; GUO *et al.*, 2008). However, little information is available on the mucosal immune response elicited by different vaccine doses, and the inappropriate dose can result in incomplete protection. A number of studies have reported the development of humoral immune responses after vaccination against IBV, most of them limited to measuring serum antibodies by ELISA, Virus Neutralization (VN) or Inhibition of hemagglutination (HI). However, these data have shown low correlation with protection against the IBV infection. The few studies that analyzed levels of antibodies anti-IBV on the respiratory mucosal surface, including lachrymal and trachea-bronchial secretions, have not yet provided correlation between local antibodies and protection against IBV (CAVANAGH, 2007). The raise of immune cell response mediated by cytotoxic T cells (CTL) in chickens against IBV infection was associated with the ceasing of clinical signs and the clearance of this virus. In addition, the transference of cytotoxic T-CD8⁺ cells from birds infected with IBV to naïve inbred chicks, provided to the adoptive transferred birds protection against this viral infection (COLLISON *et al.*, 2000). During the course of viral infections, some experiments reported that several mediators of CTLs, like Granzyme A, Granzyme B and perforins have shown a main role in the destruction of viral infected cells (GÖBEL *et al.*, 2001). SARSON *et al.* (2006) observed up-regulation of Granzyme homolog A transcripts on the beginning of Marek disease, probably related to activity of NK cells and CTLs to eliminate viral infected cells. Additionally, CTLs are typical sources of Gamma Interferon (IFN γ), cytokine associated to the development of cell-mediated immune (CMI), mainly through activation of macrophages, responsible for providing protection against intra-cellular agents (CHAMIZO *et al.*, 2001). Thus, this study was carried out aiming to evaluate the effect of different doses of attenuated IBV vaccine on the tracheal CMI and lachrymal antibody responses at 24 hours and 5 days post-challenge with IBV, correlating these immune responses with the intensity of ciliostasis, histologic lesions and viral replication in the trachea.

MATERIAL AND METHODS

Experimental design: Five groups of 14 SPF (Specific Pathogen Free) chicks (Leghorn Lineage) were used. The birds were housed in positive pressure isolators. Groups I, II and III received, respectively, full dose ($10^{4.3}$ DIE₅₀, as recommended by the manufacturer), half dose ($10^{3.99}$ DIE₅₀) and a quarter of dose ($10^{3.68}$ DIE₅₀) of attenuated H120 vaccine strain of IBV at the first day of age, by ocular-nasal route. Groups IV e V received only the vaccine diluent. At 28 days of age, groups I, II, III and IV were experimentally infected by intra-ocular and intranasal routes with $10^{4.0}$ EID₅₀/bird IBV (M41 strain). A negative control group was mock infected with SPF allantoic fluid and maintained under the same conditions. Samples of tears and serum were collected after 1, 2, 3 and 4 weeks post-vaccination (pv), and also at 24 hours (h) and 5 days (d) post-infection (pi), and stored at -20 °C. Seven chickens from each group were sacrificed at 24 hours (h) and 5 days (d) pi. Tracheal samples were collected from each group, a portion immediately frozen and kept at -70°C until processing, and the remaining was subjected to histopathological and ciliostasis analysis.

Measurement of local antibodies against IBV: Local (tears) and systemic (serum) IgG, IgA and IgM were measured from samples collected from the birds of the experimental groups by Sandwich-ELISA-Concanavalina A (S-ELISA-ConA) as recommended by BRONZONI *et al.* (2005).

Histopathology and Ciliostasis analysis: Samples of tracheas were processed and evaluated for histopathological and ciliary movement analysis as described by ANDRADE *et al.* (1992).

RNA extraction and reverse transcription: The extracted RNA from tracheal samples was treated with DNase I and reverse transcribed by standard procedures.

Viral replication: A pair of primers flanking HRV2 region at S1 gene of IBV was used to measure the level of viral replication on tracheal samples of chickens infected with IBV, by Real-time using with SYBR Green I marker (OKINO *et al.*, 2006)

Relative quantification of expression of genes related to cell-mediated immunity: The relative quantification of gene expression of CD8+ marker of cytotoxic cells, Granzyme homolog A and Gamma Interferon (IFN γ) on tracheal samples was performed by Real-time PCR using SYBR Green I. Gene expression was normalized related to 18S gene, using the birds of group V (non vaccinated and non infected) like basal gene expression (WANG *et al.*, 2006).

Statistical analysis: Differences between the groups of experimental design were analyzed by Kruskal-Wallis test followed by Dunn's, with $p < 0,05$. Correlation analysis between the tests were determined by Spearman, with $p < 0,05$.

RESULTS AND DISCUSSION

Histopathology and Ciliostasis analysis: At 5dpi, only group I (full dose of vaccine) had significant difference in histopathology and degree of ciliostasis from the infection control group (non vaccinated and infected), while the groups that received lower doses of the vaccine (II and III) presented no significant differences from infected control group (IV).

Viral replication: No significant differences for viral replication were observed between the groups infected with IBV at 24hpi. On the other hand, at 5dpi, all tracheal samples for all groups infected with IBV (vaccinated or non vaccinated) were positive for IBV, although, the degree of viral replication of group IV (non vaccinated and infected), measured by real-time RT-PCR absolute quantitation of IBV, was significantly higher than the remaining groups previously vaccinated. No significant differences for viral replication were observed between the vaccinated groups.

Significant positive correlations were found between viral replication, histopathology and ciliostasis analysis. Similar results of positive correlation had been reported for viral isolation, histopathology and ciliostasis (ANDRADE *et al.*, 1982; WINTERFIELD & FADLY, 1972).

Measurement of local and systemic antibodies against IBV: The production of antibodies to IBV was dependent of the vaccine dose administered at the first day of age. Only the full dose-vaccinated group showed significant increase of tear anti-IBV antibodies compared to the negative control group (V), from

IgG and IgA isotypes. The measurement of IgG isotype on tears showed the highest negative correlation with the three parameters of tracheal pathological alterations induced by the IBV at 5 dpi (ciliostasis, histopathology and viral replication), though, similar negative correlations were also found for IgA, but with lower correlation coefficient. Thus, the measurement of IgG on tears proved to be the more reliable marker to evaluate the protection against IBV, considering mucosal anti-IBV antibody responses. In agreement with these results, GUO *et al.* (2008) tested tracheal samples from chickens immunized twice against IBV by microarray analysis, and found that the gene expression of IgG heavy chain gene is one of the most up-regulated genes, then, they concluded that IgG secondary immune response at tracheal site is one important marker of immunological memory and this isotype must have crucial role on mucosal surface of trachea against re-exposition against this virus.

Relative quantitation of expression of genes related to immune cell response: At 24 hpi, only the full dose-vaccinated group had significant increasing of CD8⁺ marker and IFN γ transcripts compared to the unvaccinated infected group (IV), while for Granzyme homolog A, all vaccinated groups had significantly up-regulated expression compared to non-vaccinated chickens from group IV. The up-regulation of these immune-response genes (CD8⁺ marker, Granzyme homolog A and IFN γ) at 24 hpi, detected in the vaccinated groups, mainly in the full-dose vaccinated birds, probably was due to memory immune responses conferred by vaccination of one-day old chicks. At 5dpi, CD8⁺ gene expression for the vaccinated groups showed a back down regulation, leading the expression levels close to basal profile, while the non-vaccinated group was up-regulated, and had significant increasing compared to full and half-dose vaccinated groups. In this time point, the non-vaccinated infected birds showed significant increase of IFN γ transcripts just only compared to group I, and Granzyme homolog A gene expression of group IV had significant increase compared to groups I and II.

The levels of gene expression for Granzyme homolog A at 24hpi have strong negative correlation with the three parameters of tracheal pathologic changes (viral replication, histopathology and ciliostasis) at 5dpi, while for the IFN γ and CD8⁺ gene expression had strong negative correlation only with histopathology. This negative correlation indicates that the higher the values of expression of these genes at 24 hpi, the lower are the scores of tracheal lesions at 5dpi. These results suggested that the CMI responses are critical, at tracheal sites, for the IBV clearance and protection, either in vaccinated or non-vaccinated birds, but, due the memory immune responses, they are early in the first groups and delayed in the latter one.

CONCLUSION

This study showed that both significant increase of IgG and IgA isotypes in tears of previously vaccinated chickens and the expression levels of genes related to CMI (CD8⁺ marker, Granzyme A and IFN γ) were negatively correlated with one or more parameters of tracheal lesions. Moreover, the immune memory conferred at respiratory mucosal site by vaccination against IB on the first day of age, and consequently, the development of complete protection, is dependent on the vaccine dose administered.

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